BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 1/31, 1 /21, C12P 19 /04, A61K 39 /09, C12R 1 /46

A2

(11) International Publication Number: WO 98/31786

(43) International Publication Date: 23 July 1998 (23.07.98)

(21) International Application Number: PCT/GB98/00156

(22) International Filing Date: 19 January 1998 (19.01.98)

(30) Priority Data:
9700939.3 17 January 1997 (17.01.97) GB

(71) Applicant *(for all designated States except US)*: MICROBIAL TECHNICS LIMITED [GB/GB]; 20 Trumpington Street, Cambridge CB2 1QA (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WELLS, Jeremy, Mark [GB/GB]; (GB). LE PAGE, Richard, William, Falla [GB/GB]; (GB). GILBERT, Christophe, François, Guy [FR/GB]; University of Cambridge, Dept. of Pathology, Tennis Court Road, Cambridge CB2 1QP (GB).

(74) Agents: CHAPMAN, Paul, William et al.; Kilburn & Strode, 20 Red Lion Street, London WC1R 4PJ (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: NOVEL MICROORGANISMS

(57) Abstract

Novel non–invasive or non–pathogenic gram–positive microorganisms are provided which are transformed or transfected with DNA coding for one or more enzymes responsible for the production of a polysaccharide immunogen from a pathogenic bacterium. Vaccines comprising such microorganisms and their use in therapy are also provided, as are suitable DNA constructs and vectors.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistari
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Novel Microorganisms

The present invention relates to novel non-invasive or non-pathogenic Gram-positive bacterial host organisms, particularly *Lactococcus lactis* organisms, transformed with DNA coding for production of an immunogenic carbohydrate capsule. Compositions comprising such bacterial host organisms as well as their use in vaccines are also provided.

One important pathogenic organism is *Streptococcus pneumoniae*, commonly referred to as the pneumococcus. The continuing significance of *Streptoccocus pneumoniae* infections in relation to human disease in developing and developed countries has recently been authoritatively reviewed (1). That indicates that on a global scale this organism is believed to be the most common bacterial cause of acute respiratory infections, and is estimated to result in 1 million childhood deaths each year, mostly in developing countries (2). In the USA it is suggested (3) that the pneumococcus is still the most common cause of bacterial pneumonia, and that disease rates are particularly high in young children, in the elderly, and in patients with predisposing conditions such as asplenia, heart, lung and kidney disease, diabetes, alcoholism, or with immunosupressive disorders, especially AIDS. These groups are at higher risk of pneumococcal septicaemia and hence meningitis and therefore have a greater risk of dying from pneumococcal infection. The pneumococcus is also the leading cause of otitis media and sinusitis, which remain prevalent infections in children in developed countries, and which incur substantial costs.

The need for effective preventative strategies against pneumococcal infection is highlighted by the recent emergence of penicillin-resistant pneumococci. Recently it was reported that 6.6% of pneumoccal isolates in 13 US hospitals in 12 states were found to be resistant to penicillin and some isolates were also resistant to other antibiotics including third generation cyclosporins (4). The rates of penicillin resistance can be higher (up to 20%) in some hospitals (5). Since the development of

penicillin resistance among pneumococci is both recent and sudden, coming after decades during which penicillin remained an effective treatment, these findings are regarded as alarming.

For the reasons given above, there are therefore compelling grounds for considering improvements in the means of preventing, controlling, diagnosing or treating pneumococcal diseases.

It is well known that 2 major problems are encountered when trying to develop a pneumococcal vaccine that could be incorporated into a global childhood vaccination programme. These are (a) the antigenic diversity of the pneumococcus, with at least 84 immunologically distinct serotypes being known, each of which can only induce protection against its own serotype when used as a vaccine and (b) the fact that the capsular polysaccharides (each of which determines the serotype and is the major protective antigen) when purified and used as a vaccine do not reliably induce protective antibody responses in children under two years of age, the age group which suffers the highest incidence of invasive pneumococcal infection and meningitis (6). These problems also apply to other vaccine strategies based on capsule antigens (e.g. the Group B streptococcus, *Haemophilus influenza* and *Neisseria meningococcus*) although the degree of capsule type heterogeneity seems to be more restricted within these species.

The current strategy being followed in the USA with respect to the prevention of pneumococcal infections is to formulate a vaccine which contains capsular polysaccharide antigens from 23 of the most common pneumococcal serotypes. Together these strains account for approximately 90% of invasive pneumococcal isolates from children in the US and Europe and less than 70% of pneumococcal infections in Asia (7,8). In practice, the costs of manufacturing these vaccines is high, since in essence the various different capsules have to be manufactured, quality controlled and blended in appropriate quantities.

The present invention provides a means whereby the protective immunogen can be produced in a simpler bacterial fermentation (involving the growth of a non-pathogenic bacterium such as *Lactococcus lactis*, rather than the pathogenic bacterium *Streptococcus pneumoniae* itself). It also provides a means whereby more than one polysaccharide immunogen can be produced by the same bacterium, thereby reducing the number of different immunogens which have to be manufactured, quality controlled and blended. Surprisingly, we have found that using DNA comprising whole or part of the operon coding for enzymes responsible for the synthesis of a pathogenic bacterium's capsule to transform or transfect a non-invasive or non-pathogenic Gram-positive bacterial host organism results in the host organism having a polysaccharide capsule which is immunogenically and structurally identical to that normally found around the pathogenic organism. It is surprising that the use of even the whole operon would result in correct expression, secretion and assembly of the capsule given the complexity associated with these processes and the possible involvement of so called "housekeeping" genes, e.g. those involved in transport of the polysaccharide, not forming part of the operon itself, from the pathogenic organism.

There are no records in the scientific literature of the expression and/or production of polysaccharide immunogens from pathogenic bacteria in non-pathogenic Gram-positive bacteria such as *Lactococcus lactis*. Hence this invention discloses for the first time a practical method of achieving the expression and production of a polysaccharide immunogen from a pathogenic bacterium in a non-pathogenic Gram-positive organism such as *Lactococcus lactis*. Moreover the invention discloses that immune responses to the polysaccharide capsule can be elicited by polysaccharide expressor strains of such organisms.

Although the skilled person will appreciate that the invention has many possible different kinds of use, its contribution to the field of anti-capsule based vaccines and especially pneumococcal vaccines is of particular importance. Another important

possibility opened up by the invention is the provision of mucosally, e.g. nasally or orally, deliverable vaccines against immunogenic polysaccharide capsules.

Thus, in a first aspect the present invention provides a non-invasive or non-pathogenic Gram-positive bacterium transformed or transfected with DNA which codes for one or more enzymes responsible for the production of a polysaccharide immunogen from a pathogenic bacterium.

Examples of Gram-positive bacteria which can be used include *Listeria innocua, Staphylococcus xylosus, Staphylococcus carnosus, Streptococcus gordonii*, a *Lactococcus* species or a *Lactobacillus* species. One preferred embodiment of the invention uses *Lactococcus lactis*.

Alternatively, the invention can make use of an attenuated strain of a Gram-positive pathogenic bacterium, for example vaccine strains of *Listeria*, e.g. *Listeria monocytogenes*.

In a preferred embodiment the polysaccharide immunogen forms a polysaccharide capsule. One exemplified example of such a capsule is that from *Streptococcus pneumoniae*. It will be appreciated that the choice of preferred capsule serotype will be determined by locality etc. Thus, in general one may wish to express one or more of the 23 most common polysaccharide serotypes referred to above. However, even within that group, one may only need to provide expression of a smaller number to reach acceptable levels of protection in certain areas. The invention can of course be used to express other of the 84 capsule serotypes from *S. pneumoniae*, perhaps by building a "library" of expressed capsule polysaccharides which could then be mixed appropriately to produce vaccines tailored for the most common serotypes in a particular area or population.

Although the examples given herein relate to the use of *L. lactis* to produce and/or

deliver polysaccharide immunogens from *S. pneumoniae* the invention can also be applied to the production/delivery of native or recombinant polysaccharide immunogens from any pathogenic bacterium which can be usefully expressed in the innocuous host such as e.g. polysaccharide immunogens from Group B streptococci, from other species of streptococci causing disease in animals and man, from encapsulated staphylococci and other Gram-positive pathogens, or from any other organism whether Gram-positive or Gram-negative (such as invasive encapsulated strains of *E. coli, Haemophilus influenzae, Neisseria meningiditis* etc.) whose polysaccharide capsule biosynthetic genes can be expressed in organisms such as *L. lactis*.

The capsular polysaccharides of group B Streptococci (GBS) are examples of capsules which might be produced/delivered using an innocuous organism such as *L. lactis*. The GBS associated with human diseases are generally encapsulated and comprise one of nine serologically distinct capsular polysaccharides. With only minor exceptions these polysaccharides comprise glucose, galactose, N-acetylglucosamine and N-acetylneuraminic acid or sialic acid. The specific arrangement of monosaccharides into a repeating unit defines the immunological specificity of each capsule. A map of the chromosomal region of the type III GBS operon has been generated and Southern hybridisation studies with DNA probes has shown that the entire region is highly conserved among the serotypes tested (9,10). Additionally, it was discovered that the sequences of the genes flanking this region share significant homology with other bacterial genes encoding enzymes involved in the synthesis of polysaccharides. The capsules biosynthetic operons of GrpB streptococcus thus appear to be flanked at one end by genes designated cpsF and cpsE (thought to encode CMP-sialic acid synthetase and acetyltransferase respectively) and at the other end by genes thought to be involved in the cps subunit synthesis and transport/polymerisation. The biosynthetic genes of other operons can therefore be readily obtained by PCR amplification using primers based on the published sequences of the conserved genes identified in the type III cps operon. Given tha the GBS cps biosynthetic genes could be easily obtained it

may be possible for example to construct strain of *L. lactis* which express key combinations of these enzymes and produce GBS capsule polysaccharides.

The DNA used to transform or transfect the host organism can comprise either the whole or part of the operon encoding the enzymes necessary for synthesis of the polysaccharide capsule. The essential requirement is of course that enzymes coded for by the exogenous DNA together with one or more enzymes present in the host organism provide a complete synthetic pathway for the capsule.

In one embodiment exemplified below we show that DNA fragments derived from the pneumococcal type 3 cps biosynthetic operon can be cloned on a plasmid vector in *L. lactis*. Moreover, transformation of *L. lactis* with plasmids carrying fragments of the pneumococcal biosynthetic operon results in the production of a polysaccharide capsule in *L. lactis* which is antigenically and structurally identical to the capsule produced in pneumococcus. In example 1 below the capsule genes expressed in *L. lactis* carry their own promoter and translation signals. The skilled person will appreciate, however, that it would be possible to carry out the invention using other plasmids, promoters and/or translation signals, for instance those derived from the host organism. Indeed the use of constitutive or inducible lactococcal promoters recently described in the literature (11,12) will allow the level of capsule synthesis to be varied and is likely to enable higher levels of capsule to be produced by *L. lactis*. Similar results should be obtainable in other organisms using such "tailored" promoters etc.

Additionally, it would be expected that high levels of polysaccharide expression could be achieved in *L. lactis* using the inducible T7 expression system developed by Wells *et al* (13 and GB-B-2 278 358).

More than one antigen could be produced at the same time in a Gram-positive bacterium such as *Lactococcus lactis*, either by the construction of strains of the bacterium which carry the genes required for the synthesis of two or more different

capsule polysaccharides (something which has been shown to be possible in pneumococcus when different cps operons were integrated at different loci in the chromosome (14)) or by the construction of strains of *L. lactis* for instance which produce recombinant polysaccharides in which the protective epitopes from more than one serotype are present within the same polysaccharide. The engineering of capsule polysaccharides so that they contain two or more different capsule epitopes may be possible by construction of strain of *L. lactis* for example which express key combinations of the capsule biosynthetic enzymes derived from pathogenic bacteria.

The use of a non-pathogenic GRAS organism such as *L. lactis* for the production of polysaccharide antigens such as those of pneumococcus provides a means of substantially reducing the costs of producing and formulating polysaccharides as vaccines. Either high yielding strains of *L. lactis* could be used to produce the polysaccharides (which would then be purified and conjugated to a chosen carrier protein), or the cells of *L. lactis* producing the polysaccharides could be used directly as a vaccine.

Thus, in a second aspect the present invention provides a method for the production of a polysaccharide immunogen of a pathogenic bacterium which comprises the step of transforming a non-invasive or non-pathogenic Gram-positive bacterium with DNA which codes for one or more enzymes responsible for the production of the polysaccharide immunogen and/or culturing the bacterium.

As discussed above, the Gram-positive bacteria of the invention could be used to deliver the polysaccharide immunogen and so in a third aspect the present invention provides organisms of the invention for use in medicine.

In particular, the invention provides an alternative means of providing a vaccine against capsular polysaccharide antigens. Thus, in a fourth aspect the present invention provides a vaccine comprising non-invasive or non-pathogenic Gram-

positive bacteria of the invention. Such vaccines could comprise either live or killed preparations of the polysaccharide expressing organisms, e.g. *L. lactis*. In particular vaccines adapted for administration via a mucosal route are provided. Examples of such mucosal routes include nasal, oral, via the bonchus tissue, eyes, ears, rectal, vaginal, urethral or under the tongue. Particularly preferred mucosal routes include nasal and oral. The vaccines of the present invention are particularly provided for use in humans although such vaccines could also be formulated for use in animal vaccination programmes.

When formulating the organisms of the invention the skilled person can of course make use of a range of delayed release, timed release or suitably protected formulations e.g. enteric coated capsules in order to ensure that the vaccine is delivered to sites where it will be most effective e.g. Peyer's patches. In addition, the vaccines of the invention may include conventional adjuvants.

Such vaccines would utilise mucosal route immunisation and would be expected to elicit both mucosal and systemic immune responses to the polysaccharide(s) expressed. It has been shown that Gram-positive organisms such as *L. lactis* can be used as an effective delivery vehicle for protein antigens (International Patent Application No. PCT/GB 96/02580). In those studies oral or intranasal immunisation of mice with recombinant *L. lactis* expressing the antigen tetanus toxin fragment C elicited high protective level serum antibody responses to the expressed antigen.

In a fifth aspect, therefore, the present invention provides a method of vaccinating a subject against a polysaccharide encapsulated pathogenic bacterium which comprises the step of administering to the subject an effective amount of a Gram-positive bacterium of the invention.

In a further aspect the present invention provides a method of inducing mucosal immunity against a polysaccharide encapsulated pathogenic bacterium which

comprises administering to a subject an effective amount of a Gram-positive bacterium of the invention.

One of the major advantages of the mucosal route of immunisation is that it has the capacity to stimulate the mucosal immune system and thus elicit the secretion of antigen specific antibody at the mucosal surfaces of the body. As colonisation of mucosal surfaces is a prerequisite for infection by many pathogens including capsulated bacteria (e.g. pneumococcus, *Haemophilus influenzae, Neisseria meningiditis* and Group B Streptococcus agalactiae) a vaccine which elicits anti-capsule antibody at the mucosal surface might be more effective in preventing infection and also in preventing asymptomatic carriage of the organism in the population.

In other aspects the invention provides:

i) a DNA construct comprising DNA encoding one or more enzymes responsible for the production of a polysaccharide immunogen from a pathogenic bacterium;

ii) a vector comprising a construct as defined in i), e.g. a plasmid vector;

iii) the use of a construct as defined in i) in the transformation or transfection of a non-invasive or non-pathogenic Gram-positive bacterium; and

iv) the use of a bacterium of the invention in the preparation of a vaccine against a polysaccharide encapsulated pathogenic bacterium.

Preferred features of each aspect of the invention are equally applicable to each other aspect *mutatis mutandis*.

The invention will now be described by means of the following examples which

should not in any way be construed as limiting. The examples refer to the figures in which:

FIGURE 1: shows the position of primers S1, A1 and A2 on the DNA sequence of the region coding for the enzymes involved in the type 3 capsular polysaccharide synthesis (genbank, accession No. U15171 (19);

FIGURE 2: shows the sequence and key features of the pTREP cassette in pTREP;

FIGURE 3: shows the biosynthetic pathway for type 3 capsular polysaccharide (cps) in *Streptococcus pneumoniae*. Four enzymes are necessary to convert glucose-6-phosphate into the type 3 capsular polysaccharide structure found on *S. pneumoniae*. Additional functions are necessary for capsule transport and attachment;

FIGURE 4; shows the genetic organisation of the type 3 cps locus in *S. pneumoniae*. Two promoters are present (indicated P). One promoter transcribes the cps3D and cps3S genes and the other the cps3U and cps3M genes. DNA fragments CPS1 and CPS2 were generated by PCR using type 3 pneumococcal chromosomal DNA as a template and primer S1 in combination with primers A2 and A3 respectively. The primers were designed to contain BamHI restriction endonuclease sites at their 5' ends to facilitate cloning of the fragments into pTREP;

FIGURE 5: shows a schematic representation of plasmids pTREP, pTREP-CPS1 and pTREP-CPS2. MLS: macrolide, lincosamide and streptogramin B resistance determinant; T: downstream terminator. The locations of the key restriction sites are indicated and the cloned pneumococcal DNA fragments CPS1 and CPS2 (Fig. 1B above) are indicated as bold lines;

FIGURE 6: shows immunodetection of type 3 cps production by *L. lactis* with type 3 specific antisera as described in the example. (1) non-expressor strain of *L. lactis*

containing pTREP; (2) *L. lactis* cps-expressor strain UCP1619; and (3) *L. lactis* cps-expressor strain UCP1618;

FIGURE 7: shows similar proton NMR spectra for purified polysaccharide of (1) *Lactococcus lactis* expressor strain UCP1619 and (2) type 3 *Streptococcus pneumoniae*. The integration of the four hydrogen peaks (indicated A, B, C and D) gave similar ratios for the two samples of capsular polysaccharide (In 1 A:B:C:D = 10.2: 28.3: 53.8: 7.7; In 2 A:B:C:D = 13.8: 31.1: 46.2: 8.9). These results provide evidence that the structure of the cps produced by *L. lactis* is the same as that naturally occurring on type 3 *S. pneumoniae*;

FIGURE 8: shows anti type 3 cps- IgG serum antibody responses in mice immunised with a recombinant strain of *L. lactis* expressing type 3 cps. At day 21 and 35 post immunisation the mean cps specific antibody titres were significantly higher (p=0.004) than the naive group of mice. The error bars represent one standard deviation from the means. These results show that immunisation with *L. lactis* expressing cps elicits specific anti-cps serum antibody response in mice; and

FIGURE 9: shows the ^{13}C NMR spectra of purified cps from (1) type 3 *S. pneumoniae* (60h at 335K) and (2) *L. lactis* strain 1619 (44h at 320K). Both spectra were obtained on a 600Mhz machine. In both spectra the position of the carbon peaks are identical.

FIGURE 10: shows % challenge inocular of S. pneumoniae present in BAL from mice immunized with *L. lactis* 1619 as described in example 6 or from control mice 4h post-challenge with live S. pneumoniae.

FIGURE 11: shows bacterial recovery of S. pneumoniae in BAL from mice immunised with *L. lactis* 1619 as described in example 6 or control mice 4h post-challenge with live S. pneumoniae.

FIGURE 12: shows a) oligonucleotide primers used to synthesise long DNA fragments, containing the capsule biosynthesis genes from all nine serotypes of group B Streptococcus; and b) restriction endonuclease digests of the PCR products obtained from six different serotypes of GBS.

EXAMPLE 1

Cloning and expression of the *S. pneumoniae* type 3 cps biosynthetic pathway enzymes in *L. lactis*

i) Preparation of DNA fragments coding for biosynthetic pathway enzymes

Genomic DNA from *Streptococcus pneumoniae* strain WU2 which produces a type 3 polysaccharide capsule was prepared by a standard method from cells grown in BTS medium (Difco) containing glucose. The bacteria were recovered from the growth medium by centrifugation at 3000g for 10 min in a Jouan centrifuge (model CR312) and resuspended in 100µl of DNA prep buffer (10mM Tris-HCl pH 8.0, 1mM EDTA, 25% w/v sucrose) to which 10µl of 10% w/v sodium dodecyl sulphate (SDS) and 0.6µl of a 20mg/ml solution of proteinase K (Sigma) were added. The solution was incubated overnight at 37°C and then the DNA extracted twice with a mixture of chloroform, phenol and isoamyl alcohol (IAA) (24:24:2) and twice with chloroform:IAA (98:2). The genomic DNA was precipitated at –70°C for 20min by adding 0.1 volumes of 3M sodium acetate and 2 volumes of absolute ethanol. The pellet of DNA was recovered by centrifugation and resuspended with 100µl of TE (Tris-HCl pH8.0; 1mM EDTA) and precipitated a second time by adding 20µl of 4M lithium chloride and 2.5 volumes of ethanol. The purified genomic DNA was finally resuspended in TE and stored at 4°C.

DNA fragments encoding the genes required for type 3 capsule biosynthesis were then obtained by PCR using the *S. pneumoniae* genomic DNA as template and oligonucleotide primers (sense primer S1 and antisense primer A2 or A3) based on the

published sequence of the type 3 capsule locus (see figure 1 for the sequences of the cps operon and primers). The PCR sense and antisense primers were designed to include a BamHI restriction endonuclease site at their 5' end to facilitate subsequent cloning steps.

The PCR reaction mixture contained approximately 15 ng of chromosomal DNA from the WU2 strain, 20 pmol of each primer, 250μm each dNTP, 2 units of Taq polymerase and 1μl of Taq extender (Stratagene) in the Taq plus reaction buffer supplied by the manufacturer (Stratagene). After 35 cycles comprising a denaturation step (95°C for 30 sec), annealing step (60°C for 60 sec) and polymerase extension step (72°C for 180 to 240 sec) the PCR products obtained with primers S1 plus A2 and S1 plus A3 were purified and determined to be of the expected sizes (approximately 3 and 44 kb respectively) by agarose gel electrophoresis using standard methods.

ii) Construction of pTREX and pTREP

Synthetic oligonucleotides encoding a putative RNS stabilising sequence, a translation initiation region and a multiple cloning site for target genes were annealed by boiling 20μg of each oligonucleotide in 200μg of each oligonucleotide in 200μl of 1 x TBE, 150mM NaCl and allowing to cool at room temperature. Annealed oligonucleotides were extended using Tfl DNA polymerase in 1 x TF1 buffer containing 250μM deoxynucleotide triphosphates and 1.5mM $MgCl_2$ at 35°C, 45°C, 55°C and 65°C each for 1 minute followed by 10 minutes at 72°C. The sense and antisense oligonucleotides contained the recognition sites for NheI and BamHI at their 5' ends respectively to facilitate further cloning.

The resulting ds DNA was cut with NheI and BamHI and cloned between the XbaI and BamHI sites in pUC19NT7, a derivative of pUC19 which contains the T7 expression cassette from pLET1 (17) cloned between the EcoRI and HindIII sites. The resulting construct was designated pUCLEX.

The complete expression cassette in pUCLEX was then removed by cutting with HindIII and blunting followed by cutting with EcoRI before cloning into the EcoRI and SacI (blunted) sites of pIL253 to generate the vector pTREX.

Plasmid pTREP was constructed as follows: Synthetic oligonucleotides encoding the rho independent terminator from the 3' end of the penicillinase gene from *B. lichenformis*, a PUC reverse sequencing primer, a multiple cloning site for the insertion of promoters and a universal translation stop sequence (i.e. stop codons in all three reading frames) were annealed as described above. The annealed DNA fragment was cut with EcoRV and BamHI and cloned into pTREX which had been cut with Eco RI, blunted and then cut with BamI. The resulting plasmid, designated pTREP, was used for the cloning of the cps operon genes from S. pneumoniae as described below. Details of pTREP are shown in figures 2 and 5.

iii) Cloning of cps operon genes from S. pneumoniae in pTREP

The purified DNA fragments (cps1 and cps2 are shown in figure 5) were digested with BamHI using standard conditions and ligated to purified Bg1II and BamHI cut and dephosphorylated plasmid pTREP using a ligation kit (Amersham) and the conditions recommended by the manufacturer. The ligated DNA was electroporated into competent *L. lactis* as previously described (16) and transformants recovered on GM17 agar containing 5μg/ml of erythromycin to select for transformants carrying the plasmid. The resulting plasmids carrying the 3 and 4 kb DNA fragments of the S. pneumoniase type 3 cps locus were designated pTREP-CPS1 and pTREP-CPS2 respectively and are shown in figure 5. The 3 kb pneumococcal DNA fragment cloned in pTREP-CPS1 includes the cps3D and cps3S genes which encode UDP glucose dehydrogenase and the type 3 capsular polysaccharide synthetase and their associated promoter (figure 5). The pneumococcal DNA fragment cloned in pTREP-CPS2 is approximately 1 kb longer and in addition to cps3D and cps3S contains the cps3U

gene for glucose-1-phosphate uridyl transferase (figure 3). The cpsM gene was not cloned as it was expected that this enzyme would be naturally present in *L. lactis*. As shown below in example 2, this expectation was correct.

The *L. lactis* strains carrying plasmids pTREP-CPS1 and pTREP-CPS2 were designated UCP1618 and UCP1619 respectively. The production of a polysaccharide capsule by these strains can be visualised by light microscopy after Gram and nigrosin staining of thin dry smears of bacteria on glass slides.

EXAMPLE 2

The capsular polysaccharide produced by *L. lactis* is recognised by pneumococcal cps type 3 specific antisera

The *L. lactis* strains UCP1618 and UCP1619 were diluted 1:100 from a fresh overnight culture into M17 broth containing 0.5% w/v glucose (GM17) and erythromycin (5µg/ml) and grown for 3 hours at 30°C. The bacterial cultures were then concentrated approximately 10 fold by centrifugation and 5µl spotted onto a piece of nitrocellulose and allowed to dry. As a negative control a *L. lactis* strain carrying the vector pTREP was treated in the same way and applied to the nitrocellulose filter. Immunoblotting with the type 3 cps antisera was performed as previously described (12) except that dried milk (2% w/v) was used in place of bovine serum albumin as the blocking agent. The results showed that the type 3 cps antisera binds specifically to areas of filter onto which *L. lactis* strains UCP1618 and UCP1619 were spotted but not to *L. lactis* itself (negative control). As expected the antisera also detects purified pneumoccal cps by Immunoblotting (results not shown). It is apparent in Figure 6 that strain UCP1619 contains more capsular material than strain UCP1618. This result was reproducible and seen with other independently derived clones of *L. lactis* containing pTREP-CPS1 and pTREP-CPS2.

These results indicate that only the cps3D and cps3S genes of pneumococcus are necessary for type 3 cps production in *L. lactis* although the amount of capsule produced can be greatly increased by including the gene for cps3U on the same plasmid. By inference a gene encoding phosphoglucomutase (designated cps3M in figure 3) must be present in *L. lactis* and present in sufficient amounts to allow efficient capsule production in this host. However, it may be possible to further increase the yield of capsule in *L. lactis* by providing the pneumococcal cps3M gene on the same plasmid as the other capsule biosynthetic genes.

EXAMPLE 3

Proton nuclear magnetic resonance (NMR) analysis of the type 3 polysaccharide capsule produced by recombinant *L. lactis*

The capsule polysaccharide produced by *L. lactis* was purified from *L. lactis* strain UCP1619 grown overnight in MOPS buffered medium (MOPS, Alexis co., 6.7g/l pH 7.2; yeast nitrogen base, Difco, 25.11g/l; casein enzymatic hydrolysate, Sigma, 2g/l; glucose 0.5% w/v and erythromycin 5µg/ml). Protein was removed from the culture medium by adding 0.25 volumes of 100% trichloroacetic acid and centrifugation at 2000rpm for 10min in a Jouan centrifuge (model CR312). The cps was recovered by precipitation with an equal volume of acetone and centrifugation at 3000rpm for 10min in a Jouan centrifuge (model CR312). The cps from *L. lactis* was then redissolved in 5% (w/v) sodium acetate and residual protein removed by extraction with mixture of chloroform and butanol (5:1). The cps was precipitated again with an equal volume of ethanol, washed once with both ethanol and acetone and redissolved in deionised water. The cps was then freeze dried for longer term storage.

The purified cps produced by *L. lactis* strain UCP1619 and a commercial preparation of highly purified type 3 cps from S. pneumoniae (Merck Sharpe Dohme) were resuspended in heavy water D_2O water at a concentration of approximately 3.3 mg/ml

and the proton NMR spectra analysed at 320°K in a DRX 500 NMR spectrophotometer (Bruker Co. Germany).

The proton NMR spectra of native pneumococcal cps and the lactococcal-derived cps preparations are very similar. The small variations in the relative intensities of the minor peaks associated with each hydrogen group are most likely to be due to differences in the viscosity of the samples (Figure 7). Similarly the ^{13}C NMR spectra of the samples show peaks at identical positions. These results provide evidence that the molecular structures of the repeating unit of the two polysaccharide samples are the same (figure 9).

EXAMPLE 4

Investigation of immune response to isolated capsular polysaccharide and encapsulated *L. lactis*

The immune responses to recombinant *L. lactis* expressing cps and the purified cps from S. pneumoniae were compared by intrs peritoneal (i/p) inoculation of inbred strains of BALB/c mice. Groups of 5 mice were immunised s/c without adjuvant with varying doses of recombinant *L. lactis* (strain UCP619) expressing cps or equivalent amounts of the purified type 3 cps antigen from S. pneumoniae. Blood samples were collected on days 7, 21, 35 and 49 post immunisation and the anti-cps antibody levels measured by an ELISA.

For the ELISA, microtitre plates (Nunc; maxisorb) were coated with purified cps from S. pneumoniase by adding 50µg/ml solution of cps to each well and incubating for 5h at 37°C and then overnight at 4°C. The non-specific protein binding sites were blocked by adding 100µl of 1% w/v BSA in PBS to each well and incubating the plates for 1h at room temperature.

After washing the plates 3 times in washing buffer (WB; PBS; 0.05% (w/v) tween 20) the samples of sera which had been diluted in 1% w/v BSA in PBS were added to the plate (50µl/well) and incubated for 2h at room temperature. The plates were then washed 5 times in WB and incubated with the second antibody (anti-mouse IgG-alkaline phosphatase conjugate) diluted in WB containing 1% BSA (50µl/well) for 1.5h at room temperature. Finally the plates were washed 5 times with WB and incubated with the substrate nitrophenylphosphate in diethanolamine buffer and assayed using an ELISA reader at 405nm wavelength.

For each serum sample the endpoint titre which would give the same adsorbance value (405nm) as a 1:50 dilution of pooled pre-immune mouse serum was calculated using the ELISA data. A bar chart showing the mean endpoint titre of specific anti-cps antibody levels for the different experimental groups of mice on days 7, 21, 35 and 49 post immunisation is shown in figure 8.

These results show that immunisation of mice with a single dose of purified cps or *L. lactis* expressing an equivalent amount of cps elicits significant cps-specific IgG serum antibody responses. At days 21 and 35 post immunisation the mean cps-specific antibody titres of all the immunised groups of mice were significantly higher than the naïve ($p=0.004$; all statistical analyses were calculated using the non-parametric Mann-Whitney U test). At day 21 there were no significant differences ($p>0.05$) between the IgG antibody titres of the groups of mice immunised with purified cps of *L. lactis* expressing equivalent amounts of cps. At day 35 the cps antibody titres for the groups of mice given 5µg of cps (whether purified or expressed by *L. lactis*) were significantly higher than the groups immunised with the lower dose of cps ($p=0.048$). By day 49 post immunisation the cps specific serum IgG antibody titres in the immunised groups of mice were beginning to decline to levels which were not significantly different to the naive group.

EXAMPLE 5

The cloning of other pneumococcal capsule operon genes for expression in *L. lactis*

There is high homology between pneumococcal DNA sequences within ORFs (open reading frames) located upstream of the type specific cps biosynthetic operon of other serotypes. This is evident from comparison of the published DNA sequences from the capsule locus of type 3 pneumococcal isolates (18,19) and other serotypes such as type 14 (Nuijten *et al* Genbank Accession No. X85785) and 19F (20). This DNA sequence homology can be used to design primers for inverse PCR amplification of DNA fragments encoding the cps biosynthetic operon from other serotypes of pneumococcus for which the sequences are unknown (see below).

Furthermore, there is evidence in the literature that the conserved DNA sequences referred to above are also found adjacent to the capsule biosynthetic operons of other Gram-positive bacteria (21 and reference therein) suggesting that the method is applicable to a wide range of capsulated bacteria.

In the example given here, a fragment which is expected to contain the cps biosynthetic operon of pneumococcus serotype 4 has been amplified by inverse PCR. Genomic DNA from pneumococcal serotype 4 was prepared by the method described in example 1. Primers S2 and A4 were designed using the published conserved sequence referred to above and used for inverse PCR using published methods (22). A DNA fragment of approximately 13kb was amplified in the PCR reaction (30 cycles of denaturation at 94°C for 30sec; annealing at 60°C for 60sec; extension at 72°C for 8 min and a final extension period of incubation at 72°C for 10min). It is expected that the DNA sequence of this fragment will encode the cps biosynthetic enzymes of the type 4 operon.

EXAMPLE 6

1. Preparation of *L. lactis* for immunization

For the growth of *L. lactis* 1601 (with plasmid) and *L. lactis* 1619 (with capsule). Powdered M17 broth and glucose are dissolved in ddH_2O, autoclaved then cooled. Erythromycin (5μ/ml) is added to the media.

Bacteria from frozen stocks are used to inoculate 100 ml volumes of media and are cultured overnight at 30°C in a shaking incubator. The bacteria are separated from the media by centrifugation at 10,000 x g and 4°C for 10 minutes. The cells are resuspended in PBS and washed twice in PBS by centrifugation. The final suspension is estimated for colony forming units by measurement of optical density at 405nm. The concentration of cells is adjusted to that required.

2. Immunization Procedure

Lactococcus lactis 1619 was adjusted to a concentration of 5×10^9 CFU per ml and a total volume of 0.1 ml (representing 5×10^8CFU) administered by subcutaneous injection with a 26 gauge needle to each of 6BALB/c male specific pathogen free mice (aged 7 weeks). Mice were immunized on days 0, 14 and 28 with the same concentration of live bacteria. Control mice consisted of 6 untreated animals.

3. Intra Tracheal Bacterial Challenge of Mice

Bacterial challenge with *Streptoccus pneumoniae* NTCC12695 occurred on day 42. Mice (BALB/c) were sedated by intravenous saffan anaesthesia (0.15 ml; 20 mg alphadone in PBS /kg body weight; Pitman-Moore, Nth Ryde, NSW, Australia). The trachea was exposed orally and a 22.5G catheter (Terumo, Tokyo, Japan), with the needle retracted into the plastic, inserted into the tracheal opening to a distance of 0.5-0.75 cm. The needle is removed from the catheter and a 20μl volume of *S. pneumoniae* (containing 5×10^5 colony forming units) in PBS placed into the hub of the catheter. The inoculum was dispersed with two 0.3 ml volumes of air administered

by sealing a 1 ml syringe into the hub of the catheter.

4. Collection of samples following Bacterial Challenge

Mice were killed by an overdose of pentobarbital sodium administered by intraperitoneal injection. 0.2 ml of undiluted pentobarbital sodium was injected using a 26 gauge needle on a 1 ml syringe. Blood was obtained by heart puncture using a 26 gauge needle and 1 ml syringe, placed in a 1.5 ml eppendorf and allowed to clot for collection of serum. The trachea was exposed through the neck, freed by severing dorsal connective tissue, cut near the larynx and bronchoalveolar lavage (BAL) was obtained by instilling and recovering 0.5 ml of PBS into the lungs using a 20 gauge cannula with a 1 ml syringe attached and inserted into the trachea. The recovered BAL was placed in a 1.5 ml eppendorf. The BAL was assessed for the presence of bacteria by plating of 10-fold serial dilutions onto Blood Agar plates for CFU determination.

Serum was separated by centrifugation at 4°C and 450xg for 10 min (Juoan BR3.11, St. Nazaire, France) and stored at –80°C until required. 100µl BAL is placed in a cytocentrifuge and centrifuged for 10 min onto glass slides. Slides are stained with Diff Quik and differential populations of white cells are counted from 3 fields per slide and a mean % count per animal, followed by a mean % count per group determined. BAL was centrifuged for 10 min at 1000 rpm and 4°C (Juoan BR3.11, St. Nazaire, France). The supernatant is removed for storage at –80°C until required. The pellet was resuspended in 50µl PBS and 50µl methylene blue. The white cells were counted using a haemocytometer and the calculation adjusted for removal of BAL for analyses described above.

RESULTS

Animals immunized by subcutaneous injections of *L. lactis* 1619 expressing the polysaccharide capsule homologous to S. pneumoniae NTCC12695 showed enhanced

clearance of the bacteria from the bronchoalveolar region of the lungs.

Less total bacteria were recovered in the BAL of animals immunized by subcutaneous injection of *L. lactis* 1619 expressing the polysaccharide capsule homologous to the S. pneumoniae strain used to infect the lungs.

EXAMPLE 7:

Based on the published map of the chromosomal region of the type III GBS operon (1) we designed oligonucleotide primers (Fig. 12a) which were used to synthesize long DNA fragments (by PCR) containing the capsule biosynthesis genes from all nine serotypes of group B. Streptococcus. PCR primers were designed using the published sequence for the cpsF gene (EMBL database accession No. SA 19899) and the cpsC gene (EMBL database accession No. SACPSABD). DNA fragments ranging in size from 13kb to 16kb in length were amplified in the PCR reaction using genomic DNA from eight different capsule serotypes of GBS. PCR reactions were performed using the Taqplus system (Stratagene Ltd.), approx. 150 ng of genomic DNA as template and 40 pmol of each primer in the buffer recommended by the manufacturer. The reactions were incubated for 5 min at 95°C to denature the genomic DNA followed by 5 cycles of denaturation at 95°C for 30 s; annealing at 60 for 1 min; extension at 72°C for 15 min, then 5 cycles of denaturation at 95°C for 30 s; annealing at 55 for 1 min; extension at 72°C for 15 min, then 25 cycles of denaturation at 95°C for 30 s; annealing at 65 for 1 min; extension at 72°C for 15 min and a final extension period of 10 min at 72°C.

Restriction endonuclease digests of the PCR products obtained from six different serotypes of GBS showed as expected that the capsule biosynthetic operons have some fragments in common and others which are specific to the serotype (Fig. 12b). The fragments generated can be cloned and sequence or directly sequenced to identify genes involved in capsule biosynthesis. It is expected that key combinations of these

enzymes can be cloned for example into a *L. lactis* expression plasmid in order to generate innocuous strains of *L. lactis* which produce GBS capsule polysaccharides or recombinant polysaccharides in which the protective epitopes of cps from more than one GBS serotype are present.

5

REFERENCES

1. Fiber, G.R., *Science*, **265**: 1385-1387 (1994);
2. Stansfield, S.K., *Pediatr. Infect. Dis.*, **6**: 622 (1987);
3. Breiman *et al*, *Arch. Intern. Med.*, **150**: 1401 (1990);
4. Schappert, S.M., *Vital and Health Statistics of the Centres for Disease Control/National Centre for Health Statistics*, **214**:1 (1992);
5. Breiman *et al*, J. Am. Med. Assoc., **271**: 1831 (1994);
6. Douglas *et al*, *J. Infect. Dis.*, **148**: 131-137 (1983);
7. Van Dam *et al*, *Antonie van Leewenhoek*, **58**: 1-47 (1990);
8. Alonso De Velasco *et al*, *Micobiol. Reviews*, **59**: 591-603 (1995);
9. Kuypers *et al*; *Infect. Immun.* **57**: 3058-3065 (1989)
10. Rubens *et al*; *Dev. Biol. Stand. Basel, Karger,* **85**: 237-244
11. Wells, J.M. and Schofield, K.M., *Lactic Acid Bacteria: Current advances in Metabolism, Genetics and Applications* (EDS. Bozoglu, T.F. and Bibek, R.) NATO ASI series, Springer-Verlag, Heidelberg, **H98**: 37-62 (1996);
12. Waterfield *et al*, Gene, **165**: 9-15 (1995);
13. Wells *et al*, *Mol. Micobiol.*, **8**: 1155-1162 (1993);
14. Bernheimer, H.P. and Wermundsen, I.P., *J. Bacteriol.*, **98**: 1073-1079 (1969);
15. Wells *et al*, *Antonie van Leewenhoek*, **70**: 317-330 (1996);
16. Norton *et al*, *Vaccine* (in press) (1996);
17. Wells *et al*, *J. appl. Bact.*, **74**: 629-636 (1993);
18. Garcia *et al*, *Mol. Gen. Genet.*, **239**: 188-195 (1993);
19. Dillard *et al*, *J. Exp. Med.*, **181**: 973-983 (1995);
20. Guidolin *et al*, *Infect. Immun.*, **62**: 5384-5396 (1994);
21. Stingele *et al*, *J. Bacteriol.*, **178**: 1680-1690 (1996);
22. Innis *et al*, *PCR Protocols: A Guide to Methods and Applications, Academic Press Inc., San Diego, California* (1990).

CLAIMS

1. A non-invasive or non-pathogenic Gram-positive bacterium transformed or transfected with DNA which codes for one or more enzymes responsible for the production of a polysaccharide immunogen from a pathogenic bacterium.

2. A non-invasive or non-pathogenic Gram-positive bacterium as claimed in claim 1 which is *Listeria innocua, Staphylococcus xylosus, Staphylococcus carnosus, Streptococcus gordoni,* a *Lactococcus* species or a *Lactobacillus* species.

3. A non-invasive or non-pathogenic Gram-positive bacterium as claimed in claim 2 which is *Lactococcus lactis*.

4. A non-invasive or non-pathogenic Gram-positive bacterium as claimed in claim 1 which is an attenuated strain of a Gram-positive pathogenic bacterium.

5. A non-invasive or non-pathogenic Gram-positive bacterium as claimed in claim 4 which is *Listeria monocytogenes*.

6. A non-invasive or non-pathogenic Gram-positive bacterium as claimed in any one of claims 1 to 5 wherein the polysaccharide immunogen forms a polysaccharide capsule.

7. A non-invasive or non-pathogenic Gram-positive bacterium as claimed in any one of claims 1 to 6 wherein the DNA is derived from *Streptococcus pneumoniae*, Group B streptococci, other species of streptococci causing disease in animals and man, encapsulated staphylococci and other Gram-positive pathogens, encapsulated strains of *E. coli*, *Haemophilus influenzae* or *Neisseria meningiditis*, or from *Neisseria gonorrhea, Bacteriodes fragilis* or other such Gram-negative pathogens.

8. A non-invasive or non-pathogenic Gram-positive bacterium as claimed in claim 7 wherein the DNA is derived from *Streptococcus pneumoniae*.

9. A non-invasive or non-pathogenic Gram-positive bacterium as claimed in any one of claims 1 to 8 wherein the DNA comprises at least one complete operon responsible for production of the polysaccharide immunogen.

10. A non-invasive or non-pathogenic Gram-positive bacterium as claimed in any one of claims 1 to 8 wherein the DNA codes for one or more enzymes responsible for the production of two or more polysaccharide serotypes.

11. A method for the production of a polysaccharide immunogen of a pathogenic bacterium which comprises the step of transforming a non-invasive or non-pathogenic Gram-positive bacterium with DNA which codes for one or more enzymes responsible for the production of the polysaccharide immunogen and/or culturing the bacterium.

12. A method as claimed in claim 11 modified by any one or more of the features of claims 1 to 10.

13. A non-invasive or non-pathogenic Gram-positive bacterium as defined in any one of claims 1 to 10 for use in medicine.

14. A vaccine comprising non-invasive or non-pathogenic Gram-positive bacteria as defined in any one of claims 1 to 10.

15. A vaccine as claimed in claim 14 which is adapted for nasal or oral administration.

16. A method of vaccinating a subject against a polysaccharide encapsulated pathogenic bacterium which comprises the step of administering to the subject an

effective amount of a non-invasive or non-pathogenic Gram-positive bacterium as defined in any one of claims 1 to 10.

17. A method of inducing mucosal immunity against a polysaccharide encapsulated pathogenic bacterium which comprises administering to a subject an effective amount of a non-invasive or non-pathogenic Gram-positive bacterium as defined in any one of claims 1 to 10.

18. A method as claimed in claim 16 or claim 17 wherein the bacterium is administered via a mucosal route.

19. A method as claimed in claim 18 wherein the bacterium is administered nasally or orally.

20. A DNA construct comprising DNA encoding one or more enzymes responsible for the production of a polysaccharide immunogen from a pathogenic bacterium.

21. A DNA construct as claimed in claim 20 modified by any one or more of the features of claims 9 and 10.

22. A vector comprising a DNA construct as defined in claim 20 or claim 21.

23. A vector as claimed in claim 22 which is a plasmid vector.

24. The use of a construct as defined in claim 20 or claim 21 or a vector as claimed in claim 22 or claim 23 in the transformation or transfection of a non-invasive or non-pathogenic Gram-positive bacterium.

25. The use as claimed in claim 24 modified by any one or more of the features of claims 2 to 8.

26. The use of a non-invasive or non-pathogenic Gram-positive bacterium as defined in any one or claims 1 to 10 in the preparation of a vaccine against a polysaccharide encapsulated pathogenic bacterium.

27. The use as claimed in claim 26 wherein the vaccine is adapted for nasal or oral administration.

```
      670         680         690         700    sense primer S1 720
TATGCATTGG TTATTGGATG TGGTTTATCG TGAAGACCAT CATCAGACCC TGGATAAACG
ATACGTAACC AATAACCTAC ACCAAATAGC ACTTCTGGTA GTAGTCTGGG ACCTATTTGC

      730         740         750         760         770
AGCTGCTTTT AACCTTAATC TTATCGAAAA ATGTGTTTAT ATTTTCTAAA ..........
TCGACGAAAA TTGGAATTAG AATAGCTTTT TACACAAATA TAAAAGATTT ..........

                  980         990        1000        1010        1020
.......... TGCTTTTTAA ATAAAGTGAG AATATTAATA ATGCAGAGAA AGAGGACTGT
.......... ACGAAAAATT TATTTCACTC TTATAATTAT TACGTCTCTT TCTCCTGACA

      1030    gene cps3D  1050        1060        1070
AGTAAAATGA AAATTGCCAT TGCAGGAAGT GGTTATGTAG GTCTGTCTTT ..........
TCATTTTACT TTTAACGGTA ACGTCCTTCA CCAATACATC CAGACAGAAA ..........

                  3440        3450     gene cps3S 3470        3480
.......... GATGTTCTGA TGATTTAGGG TGGGGAACTA GGAATTTAAC AGAGTGAGAT
.......... CTACAAGACT ACTAAATCCC ACCCCTTGAT CCTTAAATTG TCTCACTCTA

      3490        3500        3510        3520        3530
AAATAGTAGT GCGTATATAG AGTATTTACT CAGAGTATTA ATTGATTTTT ..........
TTTATCATCA CGCATATATC TCATAAATGA GTCTCATAAT TAACTAAAAA ..........

                  3620        3630        3640        3650        3660
.......... CTTGTTTTTC ATTATATTAG GGATTATTGT TGAAGTTTTG TTTTAAGGCT
.......... GAACAAAAAG TAATATAATC CCTAATAACA ACTTCAAAAC AAAATTCCGA

      3670        3680        3690        3700        3710        3720
CATTGTCATC TGTAGTGGCT CACTTCAGAC GTAAGGGTCA TATTTTAATG TGAAAAGAGT
GTAACAGTAG ACATCACCGA GTGAAGTCTG CATTCCCAGT ATAAAATTAC ACTTTTCTCA
                        reverse primer A2
      3730        3740        3750        3760        3770        3780
GTTAAAAGAT TAATCACTTA TATTATTTTA ATAGAAATAG TGTAAGGAAT TGTTATGAAA
CAATTTTCTA ATTAGTGAAT ATAATAAAAT TATCTTTATC ACATTCCTTA ACAATACTTT

    gene cps3U  3800        3810        3820        3830
AAAGTAAAAA AAGCTGTTAT TCCTGCTGCA GGGCTGGGCA CACGATTTTT ..........
TTTCATTTTT TTCGACAATA AGGACGACGT CCCGACCCGT GTGCTAAAAA ..........

      4630        4640        4650        4660    gene cps3U  4680
.......... ACGTTATTGC ACTTGGTAAG CAATTGGAGA AGCTAGATGA CTGTTCGTCA
.......... TGCAATAACG TGAACCATTC GTTAACCTCT TCGATCTACT GACAAGCAGT

      4690        4700        4710        4720        4730        4740
AGTGGACACC TATGAATTGT ATAGAAAGTT ATCAAAAATG GCTAAATGTC CCTGATCTTC
TCACCTGTGG ATACTTAACA TATCTTTCAA TAGTTTTTAC CGATTTACAG GGACTAGAAG
reverse primer A3
```

Appendix 1 : Position of primers S1, A1 and A2 on DNA sequence of region coding for the enzymes involved in the type 3 capsular polysaccharide synthesis (genbank, accession number U15171 ; Dillard *et al.*, 1995)

FIG.1

Sequence and key features of the pTREP cassette in pTREP

```
ATCGGTACCTCAAGCTCATATCATTGTCCGGCAATGGTGTGGGCTTTTTTTGTTTTAGC
    KpnI                    terminator sequence

GGATAACAATTTCACACAGGAGAATTCGCGAGATCTTCGACTAGTGTTTAAACATTA
reverse sequence primer   EcoRI     BglII                  UTS

ATTAAGCCCGGGGGATCTGGGAGACCACAACGGTTTCCCACTAGAAATAATTTTGTTT

                          Start
AACTTTAGAAAGGAGATATACGCATGCAGGATATCTCTAGAATGGATCCGGCTGCTAA
     Shine Dalgarno    SphI   EcoRV          BamHI

CAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAAC

CCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCC
        T7 terminator

GGATGACCTGCAG
        PstI
```

FIG. 2

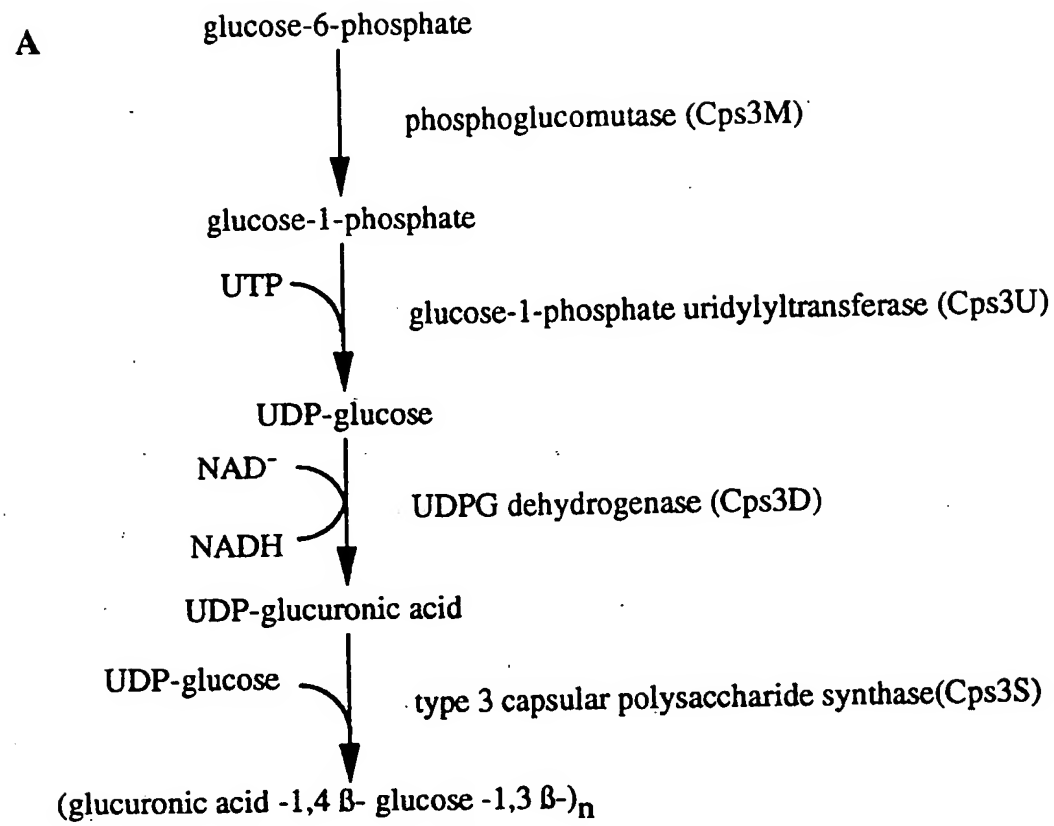

A
glucose-6-phosphate
phosphoglucomutase (Cps3M)
glucose-1-phosphate
UTP
glucose-1-phosphate uridylyltransferase (Cps3U)
UDP-glucose
NAD-
NADH
UDPG dehydrogenase (Cps3D)
UDP-glucuronic acid
UDP-glucose
type 3 capsular polysaccharide synthase(Cps3S)
(glucuronic acid -1,4 ß- glucose -1,3 ß-)n

FIG. 3

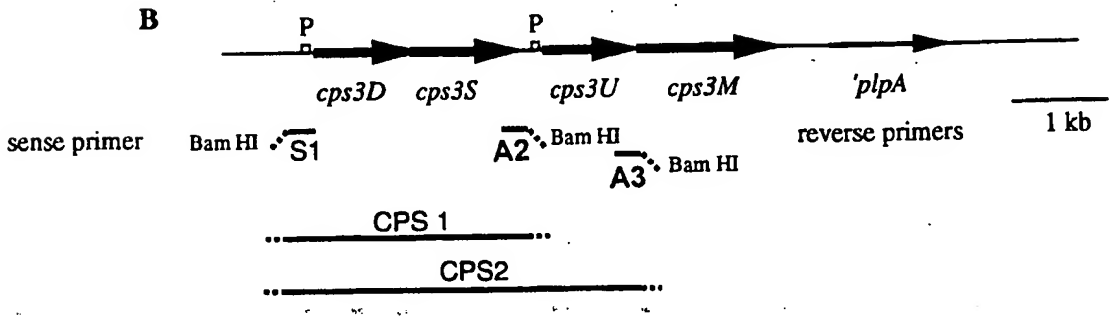

B
P
P
cps3D
cps3S
cps3U
cps3M
'plpA
1 kb
sense primer
Bam HI
S1
A2
Bam HI
A3
Bam HI
reverse primers
CPS 1
CPS2

FIG. 4

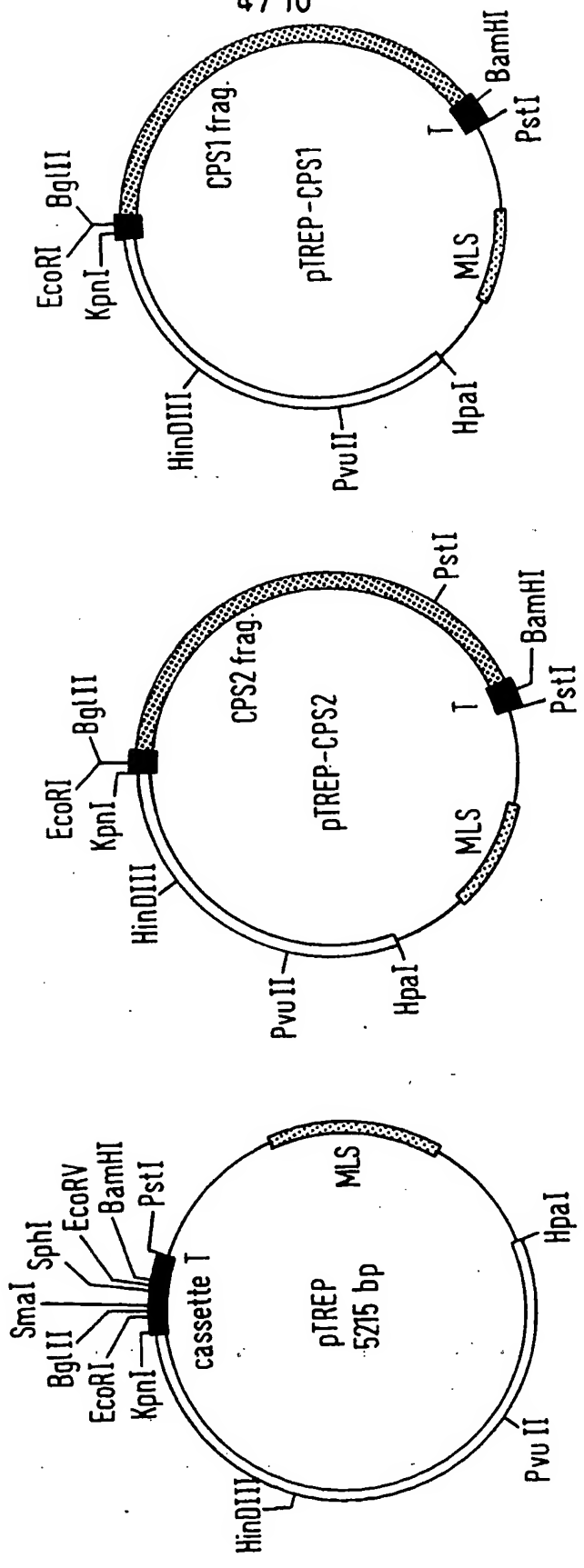

CPS1 frag.
pTREP-CPS1
BamHI
PstI
T
MLS
HpaI
PvuII
HindIII
EcoRI
KpnI
BglII
CPS2 frag.
pTREP-CPS2
PstI
BamHI
PstI
T
MLS
HpaI
PvuII
HindIII
EcoRI
KpnI
BglII
pTREP
5215 bp
MLS
HpaI
PvuII
HindIII
cassette
T
EcoRI
KpnI
BglII
SmaI
SphI
EcoRV
BamHI
PstI

FIG. 5

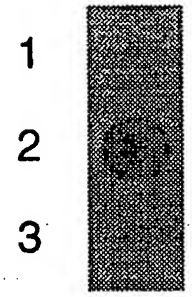


FIG. 6

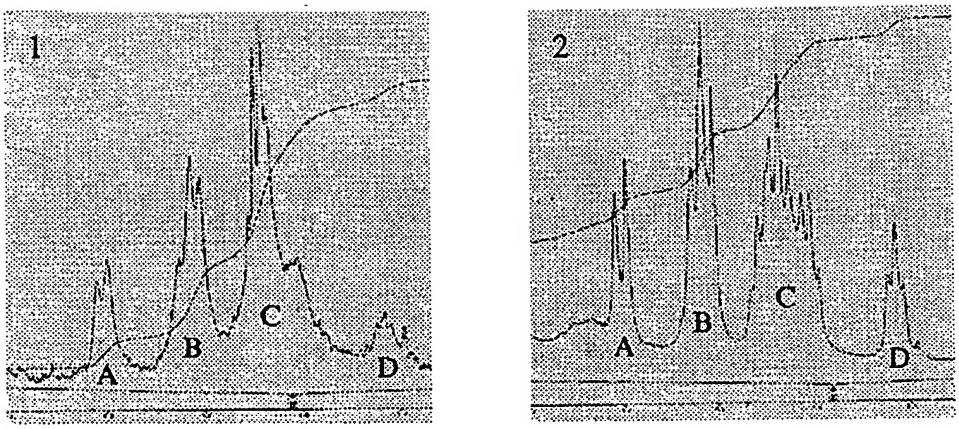
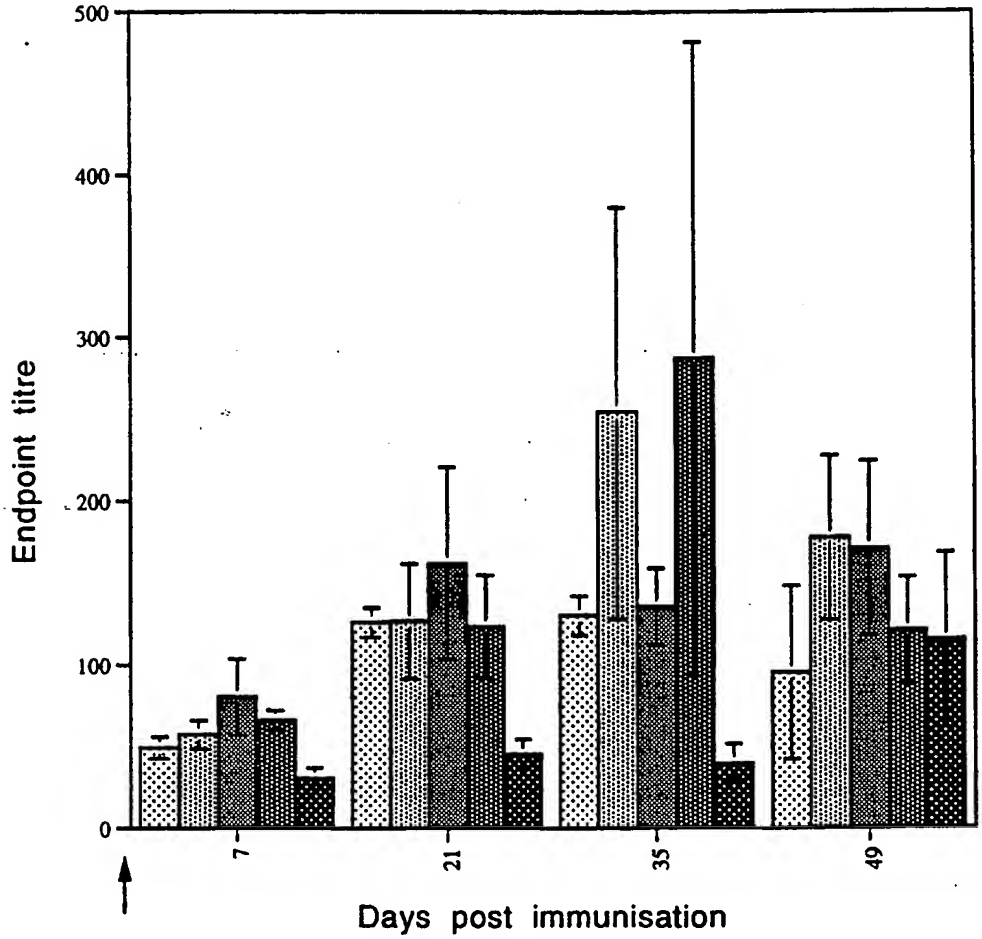


FIG. 7



Key

- 0.5 µg of purified type 3 polysaccharide from *S. pneumoniae*
- 5.0 µg of purified type 3 polysaccharide from *S. pneumoniae*
- *L. lactis* expressing the equivalent of 0.5 µg of type 3 cps
- *L. lactis* expressing the equivalent of 5.0 µg of type 3 cps
- Naive mice

FIG. 8

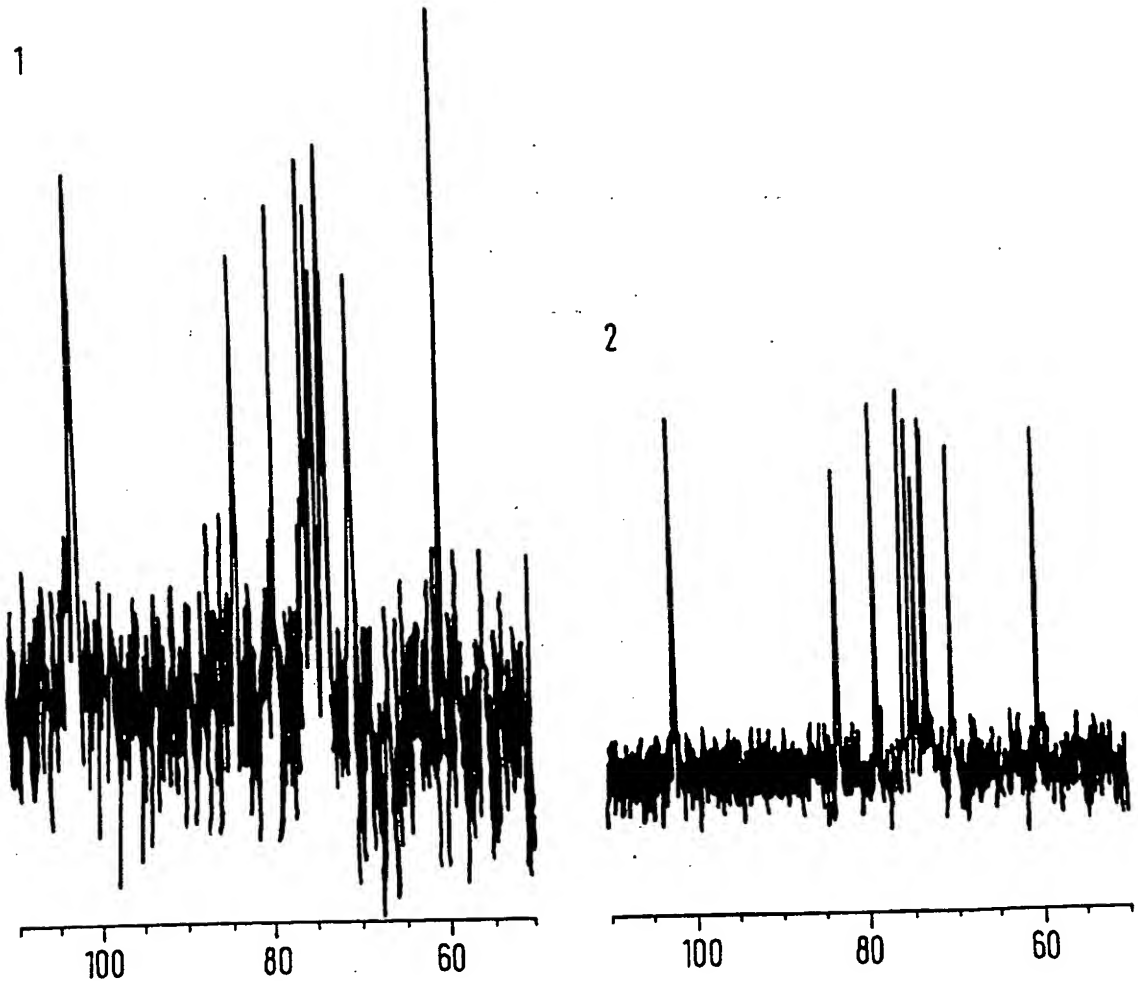
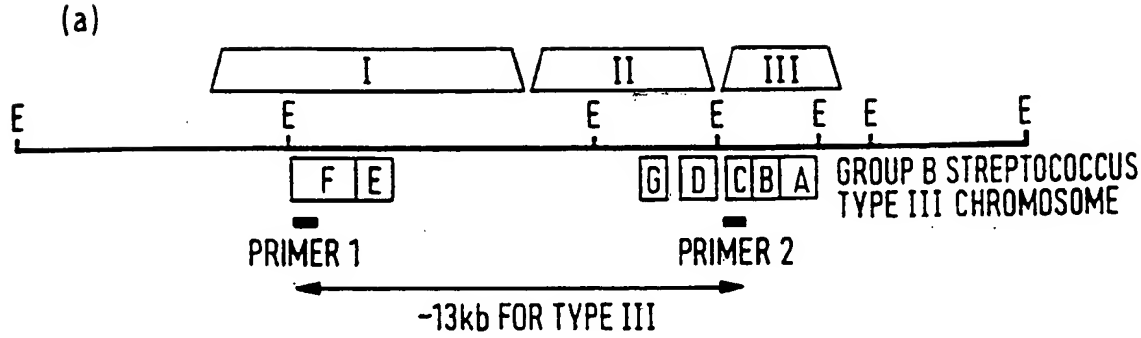


FIG. 9


(a)
I
II
III
E
E
E
E
E
E
E
F
E
G
D
C
B
A
GROUP B STREPTOCOCCUS
TYPE III CHROMOSOME
PRIMER 1
PRIMER 2
~13kb FOR TYPE III

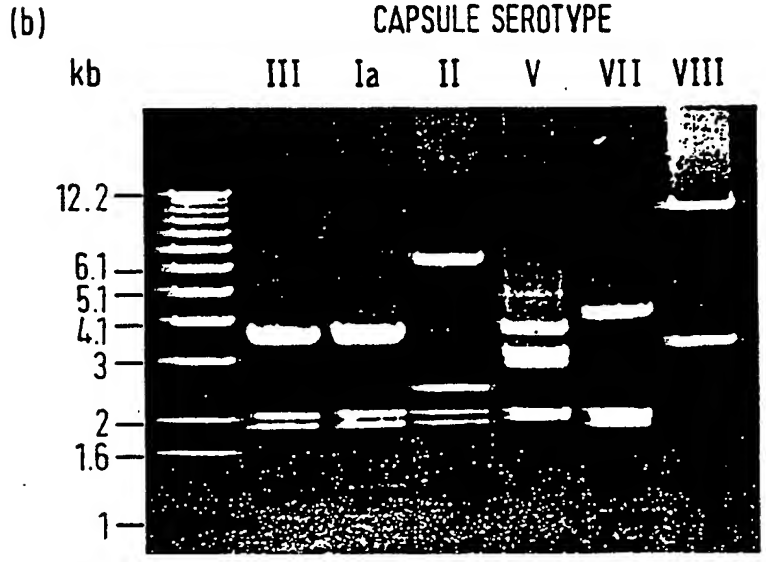
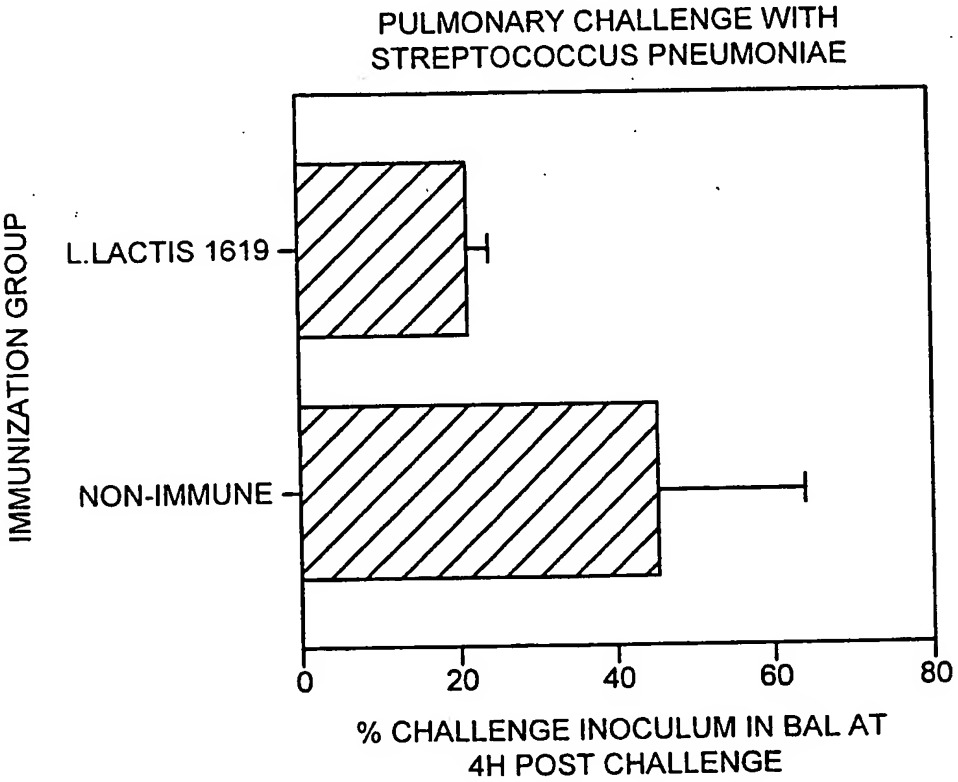

(b)
CAPSULE SEROTYPE
kb
III
Ia
II
V
VII
VIII
12.2
6.1
5.1
4.1
3
2
1.6
1

FIG. 10

RESULTS

MEASUREMENT OF BACTERIA RECOVERED IN THE BAL AT 4H POST-CHALLENGE WITH LIVE STREPTOCOCCUS PNEUMONIAE NTCC12695 EXPRESSED AS A PERCENTAGE OF THE INITIAL INOCULUM INSTILLED.

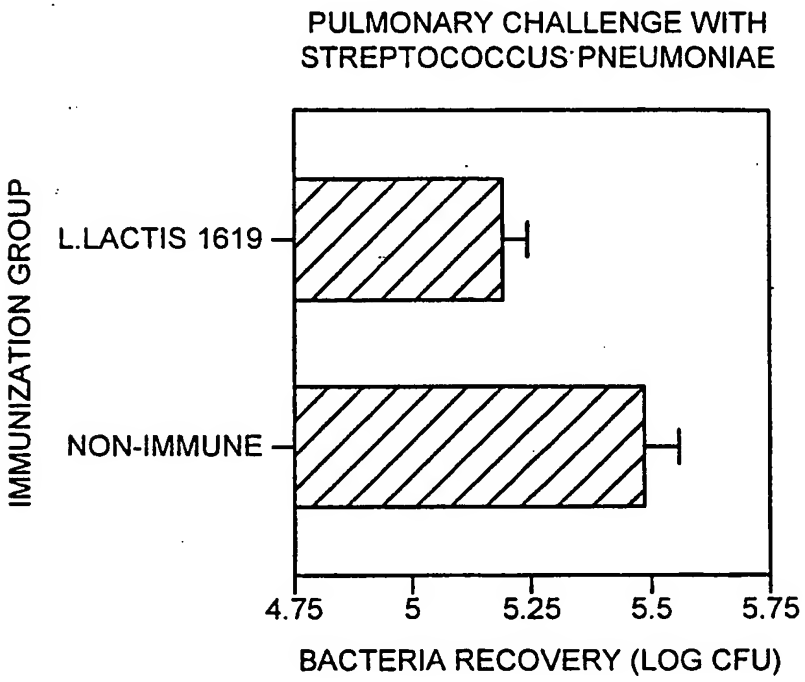


INTERPRETATION

ANIMALS IMMUNIZED BY SUBCUTANEOUS INJECTIONS OF L.LACTIS 1619 EXPRESSING THE POLYSACCHARIDE CAPSULE HOMOLOGOUS TO S.PNEUMONIAE NTCC12695 SHOWED ENHANCED CLEARANCE OF THE BACTERIA FROM THE BRONCHOALVEOLAR REGION OF THE LUNGS.

FIG. 11

STREPTOCOCCUS PNEUMONIAE NTCC12695 RECOVERED IN THE BAL AT FOUR HOURS POST PULMONARY CHALLENGE WITH 7.5 x 10^5 CFU (LOG_{10}5.88) LIVE BACTERIA.



INTERPRETATION

LESS TOTAL BACTERIA WERE RECOVERED IN THE BAL OF ANIMALS IMMUNIZED BY SUBCUTANEOUS INJECTION OF L.LACTIS 1619 EXPRESSING THE POLYSACCHARIDE CAPSULE HOMOLOGOUS TO THE S.PNEUMONIAE STRAIN USED TO INFECT THE LUNGS.

FIG. 12